#17 Declaration 1.132

Attorney Docket No. 10015

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: John Howard		Art Unit: 1638
Serial No.: 09/786,960		
Filed: March 12, 2001		Examiner: M. Ibrahim
For: COMMERCIAL PRODUCTION OF LACCASE IN PLANTS		

DECLARATION OF DR. ELIZABETH HOOD UNDER 37 C.F.R. §1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, Elizabeth Hood, hereby declare and state:

1. I am a Vice President of ProdiGene, Inc., the assignee of the above-identified application. I am Director of Industrial Proteins for ProdiGene. I am also an adjunct Professor in the Department of Biology and Department of Biochemistry and Biophysics at Texas A&M University as well as faculty at the University's Molecular and Environmental Plant Science program. Previously, I was Director of Cell Biology for plant production of therapeutic proteins at Pioneer Hi-Bred International, Inc. and before that was Assistant Professor of Biology at Utah State University, and researcher at Swedish University of Agricultural Sciences
2. I have a Ph.D. in Plant Biology from Washington University in St. Louis, and a Master of Science degree in Botany from Oklahoma State University. I have been

CERTIFICATE OF MAILING (37 C.F.R. § 1.8(a))

I hereby certify that this document is being deposited with the United States Postal Service on the date shown below with by Express Mail Post Office-to-Addressee, Express Mail No. EU74292792US in an envelope addressed to the Assistant Commissioner for Patents, U.S. Patent & Trademark Office, Washington, D.C. 20231.

June 13, 2003
Date

Patricia A. Sully

involved in the science of plant biology including expression of proteins from exogenous nucleotide sequences in plants for twenty years.

3. I have read the office action dated January 15, 2003 and understand the Examiner is questioning whether levels of expression of laccase in plants above 0.01%, as shown in the example in the application, can be achieved using the methods described in the application.

4. Attached to this Declaration is a publication in *Plant Biotechnology Journal* (Hood et al., "Criteria for high-level expression of a fungal laccase gene in transgenic maize" *Plant Biotechnology Journal* (2003) 1: pp129-140) that describes an experiment I was involved in, in which the processes described in the above-identified patent application were used to introduce heterologous laccase-encoding nucleotide sequences into maize. As discussed under the Results at page 132 of the publication, and summarized in Table 1, we achieved expression levels of 0.8% total soluble protein using those processes in T_1 seed.

5. Also attached to this Declaration is a publication now in press which will shortly appear in *Applied Microbiology and Biotechnology* (Bailey et al., "Improved recovery of active recombinant laccase from maize seed" *Applied Microbiology and Biotechnology* in press, 2003). This document discusses experiments I have been involved in, in which we used the processes described in the above-identified patent application to express laccase in plants. The details are set forth in the article. We followed the procedure as outlined in the present application and transformed maize with a laccase construct having a seed-preferred promoter and a signal sequence. We measured laccase expression in the transformed plants at 0.8% total soluble protein. (See the discussion of Results on page 6 of the journal article.) We selected highly expressing T_1 lines and backcrossed these plants into plants having favorable agronomic traits (See page 4 of article). Using the assays described in the article, we were able to determine that laccase was expressed in the plant at levels of at least about 10% total soluble protein.

6. I also have been involved in experimentation in which we attempted to express laccase in plants other than to the plant seed, or other than to cell wall. We achieved either low expression levels or the plants died, as is discussed in page 138 of the *Plant Biotechnology Journal* article attached.
7. I understand the Examiner also is concerned whether transformation of the laccase DNA into maize reflects that other plant species may be transformed as well. As one skilled in the art, I would accept transformation of maize as a satisfactory showing that transformation can occur of the DNA described into other plant species. Transformation of monocots was accomplished beginning in 1990, and dicots have been transformed since 1983. Over 50 different plant species have now been successfully transformed. When transformation is accomplished, I fully expect that there would be no obstacles to transformation in other plant species.

I further declare that all statements made herein are of my own knowledge and are true and that all statements made on information and belief are believed to be true; and further that all statements made herein were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this application or any patent issuing hereon.

Dr. Elizabeth Hood

Dr. Elizabeth Hood

Dated: 6-13-03

Plant Biotechnology Journal (2003) 1, pp. 129–140

Criteria for high-level expression of a fungal laccase gene in transgenic maize

Elizabeth E. Hood*, Michele R. Bailey, Katherine Beifuss, Maria Magallanes-Lundback, Michael E. Horn, Evelyn Callaway, Carol Drees, Donna E. Delaney, Richard Clough and John A. Howard

ProdiGene Inc., 101 Gateway Blvd., Suite 100, College Station, TX 77845, USA

Received 5 June 2002;
revised 25 November 2002;
accepted 10 December 2002.
Correspondence: 1112 Finney, College Station, TX 77845, USA (fax +1 979 693 9234; e-mail LHA105@aol.com)

Keywords: germplasm, high expression, industrial enzyme, laccase, transgenic maize.

Summary

Expression of industrial enzymes in transgenic plants offers an alternative system to fungal fermentation for large-scale production. Very high levels of expression are required to make the enzymes cost-effective. We tested several parameters to determine the best method for achieving high levels of expression for a fungal laccase gene. Transgenic maize plants were generated using an *Agrobacterium*-mediated system. The molecular parameters that induced the highest expression were the maize embryo-preferred globulin 1 promoter and targeting of the protein to the cell wall. Two independent transgenic events that yielded multiple clonal plants were characterized in detail. Independent transgenic events 01 and 03 contained two or one copies of T-DNA, respectively. Plants derived from a single transgenic event varied in expression level, and the variation in expression levels was heritable. Within the seed, expression in these plants was primarily within the embryo, and was associated with seed browning and limited germination. High oil germplasm was used to increase germination, as well as to assist in increasing expression 20-fold in five generations through breeding and selection.

Introduction

Plants are being used by a number of groups to develop their potential as biofactories for the production of proteins at commercial levels for applications as diverse as pharmaceutical treatments and textile bleaching (Hood and Howard, 1999; Hood and Jilka, 1999). Pharmaceutical and vaccine production in plants has several advantages, in that the material contains no contaminating organisms and can be directly consumed (Hood and Howard, 1999; Hood and Jilka, 1999). The major issue for the production of industrial enzymes in transgenic plants is the requirement for quite high expression levels to make them economically viable. Considering the large market opportunity for industrial enzymes and their environmentally friendly benefits, it is well worth the effort of generating an efficient production system.

Enzymes fall into several categories, which include hydrolases, transferases, oxidoreductases, lyases, isomerases and ligases (Lehninger, 1970), only a few of which are routinely utilized in industrial applications. Hydrolases are by far the most commonly used industrial enzymes. These enzymes, for example amylases and proteases, are used in numerous food processing applications and detergents. Other enzyme classes will be useful in industry when they become less expensive and thus available for testing in various processes. Oxidation/reduction (redox) enzymes are on the threshold of such a market entry.

Lignin is a biopolymer of plants that is a major component of secondary cell walls (Whetten and Sederoff, 1995). This complex polymer is formed from oxidized phenolics produced through the action of oxidase and peroxidase enzymes. The use of cell wall materials such as wood, wheat straw or maize stalks and leaves as a source of fibre, fuel or feed requires the degradation or modification of lignin. Currently, the processes which disrupt and reform lignin bonds are generally chemical, and can be highly polluting. Improved processes with less pollution are being sought.

In this regard, enzymes secreted from white rot fungi can be utilized to modify lignin. One example of such an enzyme is laccase (p-diphenoloxidase; EC 1.10.3.2) an oxidoreductase (Rheinhammar, 1984) that contains copper ions to transport and catalyse the one-electron oxidation of a variety of

substrates including phenolic hydroxyl groups. The enzyme then reduces O_2, yielding phenolic radicals and water as the reaction products. Because the activity of this oxidase is directed towards aromatic groups, applications of this enzyme include activities such as bleaching pulp, hair, skin and fibres (textiles), as well as condensing aromatic monomers into novel chemicals.

One of the major roadblocks to the use of laccase in these industries is the availability of an inexpensive large-scale source of the enzyme. In an effort to produce large amounts of laccase for industrial applications, a plant expression system is being utilized (Hood *et al.*, 1997, 1999; Hood, 2002). This system employs transgenic *Zea mays* L. as a biofactory. In this work, the *Trametes versicolor* laccase I gene (Ong *et al.*, 1997) was placed under the control of maize promoter elements to induce high levels of protein. The experiments reported here demonstrate that we have achieved a high-level expression of the active protein. The protein exhibits characteristics which are substantially equivalent to the native fungal enzyme (M. Bailey, unpublished data). For enzymes such as fungal laccases, whose activity is focused on the generation of free radicals, their location within the transgenic plant is critical because of the influence of active oxygen in plant metabolism. Several important factors that have an impact on expression must be considered, including location within specific tissues and within particular subcellular compartments to insulate the plant tissues from the activity of the enzymes. We have tested different promoters and targeting sequences in order to optimize plant-based expression and to minimize their effects on plant health. In spite of these precautions, effects on plant health were observed and a novel germplasm was employed to counteract these effects.

Materials and methods

Plant expression vectors

The laccase gene (EMBL accession no. U44430) was placed under the control of a maize constitutive promoter, a maize embryo-preferred promoter (Belanger and Kriz, 1991), or a maize endosperm-preferred promoter (GenBank accession no. AF090447, nucleotides 1–867) (Figure 1). To target the laccase gene to the cell wall, the barley alpha amylase signal sequence (BAASS, Rogers, 1985) was used in constructs PGN7699, PGN7718, PGN8908, PGN8988, PGN8996, PGN9001 and PGN9067. To target the laccase protein to the endoplasmic reticulum (ER), a DNA sequence encoding KDEL (lys-asp-glu-leu) was synthesized as an oligonucleotide and attached through PCR to the 3′ end of the laccase sequence in constructs PGN7699 and PGN9001. In PGN8915, the protein was cytoplasmically targeted and contained no additional sequences other than the promoter and the laccase I gene. The plasmids containing the barley alpha amylase signal sequence were produced by ligating oligomeric sequences encoding the BAASS sequence to the 5′ end of the laccase gene, and then the entire sequence was amplified by PCR and cloned. The sequencing of individual clones followed and confirmed the presence of the expected nucleotides. An individual clone from each vector construction was chosen for further manipulations. All target protein

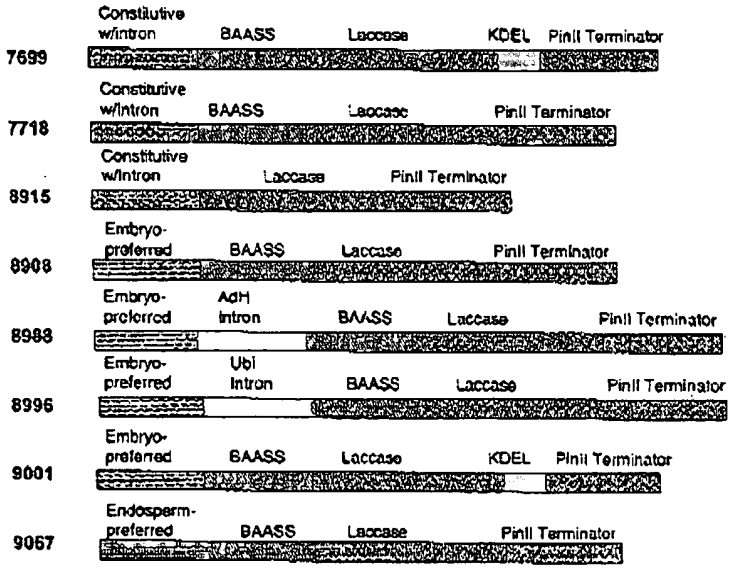

Figure 1 Plant transcription units (PTUs) used to generate transgenic plants expressing the *Trametes* laccase I gene. The ProdiGene plasmid number for each construct is shown to the left of each unit. Promoters and targeting criteria for each construct are as indicated. BAASS = barley alpha amylase signal sequence; KDEL = lysine, aspartic acid, glutamic acid, leucine as the endoplasmic reticulum retention sequence; *PinII* = protease inhibitor II gene from potato.

expression cassettes contained the terminator from the proteinase inhibitor II gene (An *et al.*, 1989).

To generate plasmids PGN7699 and PGN7718 (Figure 1) intermediate vectors with BAASS:: laccase were cut with *Nco*I and *Hpa*I, and ligated into a plasmid containing the PGNpr1 promoter and *Pin*II terminator. The entire transcription unit was cut from this latter vector with *Nhe*I and *Not*I and ligated into a recipient vector containing the 35S promoter with the PAT (phosphinothricin amino transferase) selectable marker between the left and right borders of *Agrobacterium tumefaciens*. For plasmid PGN8908 (Figure 1) the same procedure was employed, and the PGNpr1 promoter of the intermediate vector removed, substituting the PGNpr2 embryo-preferred promoter. The PGNpr2 promoter was removed from its parent vector using *Hin*dIII and *Nco*I, and was ligated into the T-DNA vector cut with the same restriction enzymes.

For plasmids PGN7699 and PGN9001, the nucleotides for the amino acids lysine, aspartic acid, glutamic acid and leucine (KDEL) were added to the 3′ end of the laccase gene by PCR amplification using a reverse primer containing the KDEL sequence. The entire coding sequence of PGN9001 was then put into the intermediate vector and completed similarly to plasmid PGN8908.

Included in all vectors was the maize-optimized *pat* gene from *Streptococcus hygroscopicus* (White *et al.*, 1992) expressed from the CaMV 35S promoter. The plant expression units were between T-DNA borders in the final cloning vector pSB11 from Japan Tobacco (Hiei *et al.*, 1994; Ishida *et al.*, 1996). The vector was mated into *Agrobacterium tumefaciens* strain LBA4404 containing the super binary vector pSB1 (Hiei *et al.*, 1994). After mating, the co-integrated vector pSB111 was isolated and electroporated into EHA101 (Hood *et al.*, 1986) and the resulting strain, EHA101(pSB111), used for plant transformation.

Gene transfer into maize

Immature embryos of Hill maize (Armstrong *et al.*, 1991) were treated with log phase *Agrobacterium tumefaciens* EHA101(pSB111) (OD = 0.5), essentially as previously described by Ishida *et al.* (1996). Embryogenic callus cultures were selected on 3 mg/L bialaphos, purified from the herbicide, Herbiace (gift of H. Ishikawa, Japan). In some cases, 5 μM bialaphos medium was made using purified bialaphos from Duchefa Biochemie BV. Regeneration of transgenic plants from the callus was as previously described (Hood *et al.*, 1997). Seedlings were transplanted into soil in the greenhouse and allowed to flower and produce seed through hand-pollinations with pollen from either Illinois High Protein-90 (Dudley and Lambert, 1992) or inbreds from various sources.

Extraction of corn seed

Five T_1 seeds were pulverized individually and homogenized with 20 mM sodium acetate, pH 5.0, containing 0.05% Tween-20 (SAT) for enzyme assay analysis. For pooled seed samples, 50 seeds were ground together in a coffee grinder and separate aliquots were extracted as for the individual seed samples. Extraction was routinely performed with a 1 : 2 ratio of seed tissue to buffer. Extracts were centrifuged for 10 min at 10 000 ***g*** to pellet the cell debris and the supernatant was placed in a fresh tube. Protein precipitated by the copper treatments (see below) was pelleted by centrifugation for 10 min at 10 000 ***g*** and the supernatant was transferred to a fresh tube.

Copper treatment of extracts

Copper treatment was carried out on clarified supernatant by adding (final concentrations) 10 mM $CuSO_4$ and 0.5 M NaCl from concentrated stock solutions prepared in H_2O. Samples were incubated in the treatment solution, typically at either room temperature or 50 °C, for ≈ 1 h. Incubation with metal salts causes up to 90% of the total soluble protein to precipitate (Bell *et al.*, 1983). Therefore, activated solutions were centrifuged for 10 min at 10 000 ***g*** to remove this precipitate, and then analysed for laccase activity using a microtitre plate assay. Samples containing chloride salts were diluted to less than 50 mM chloride or dialysed before analysis of enzyme activity.

Laccase microtitre plate activity assay

One to 10 μg of soluble corn protein was analysed in SAT buffer with 4.5 mM ABTS (2,2′-azino-*bis*-3-ethylbenzthiazoline-6-sulphonic acid) substrate (Sigma) at 25 °C and the absorbance was measured at 420 nm on a Spectramax Plus (Molecular Devices). Laccase activity was determined by comparison with purified recombinant *Trametes* laccase I from *Aspergillis* (gift of Genencor International). Total soluble protein (TSP) was measured using the Bradford (1976) method with microtitre assay conditions and reagents from Bio-Rad.

Western analysis

Laccase samples were analysed by Western blot according to the method outlined in Hood *et al.* (1997) using laccase antibodies produced in rabbit. Seed extracts from the plants generated from PGN8915 and PGN9067 were concentrated ~25-fold and from PGN7699, PGN8988 and PGN8996 ~10-fold before Western analysis.

DNA hybridization analysis

Young leaf tissue was frozen in liquid nitrogen and DNA was extracted using a Qiagen plant DNA isolation kit (Qiagen Inc., Valencia, CA). The DNA was cut with restriction endonucleases and fractionated on an agarose gel (0.8%) and subsequently transferred to nylon membranes. Blots were hybridized with a denatured DNA probe fragment that was labelled with ^{32}P-dCTP. Pre-hybridization and hybridization (Southern, 1975) were carried out in a heat-sealed plastic bag in a shaking water bath for 2 h or 16 h, respectively, at 65 °C. Blots were washed twice in a 2× SSC, 0.1% SDS solution for 5 min each at room temperature, then twice in a 1× SSC, 0.1% SDS solution at 65 °C for 15 min each, then in 0.1× SSC, 0.1% SDS at 65 °C twice for 15 min each. DNA from two transgenic lines from the 8908 plasmid were analysed. Probes comprised the laccase gene.

In situ tissue staining

Dry corn seed was soaked overnight (16 h) in sterile reverse osmosis (RO) water at 4 °C. Seeds were cut length-wise with a razor blade into numerous sequential sections ≈ 1 mm in thickness. To detect laccase activity, these sections were stained with a 0.68-mM (first dissolved in DMSO) solution of DAF (diaminofluorene) in 20 mM sodium acetate buffer, pH 5.0 with 1% DMSO. Sections were stained for 30 min. To detect lignin, sections were stained with a 1% solution of phloroglucinol in 25% concentrated HCl/75% ethanol (95%). Tissues were stained for 30 min and the pink colour recorded. All stained tissues were photographed on a Leica M6 dissecting microscope using Tungsten 160T film.

Results

Construction of expression cassettes and generation of transgenic plants

The native fungal laccase I gene from *Trametes versicolor* (Ong *et al.*, 1997) was engineered into an expression vector that contained either the constitutive PGNpr1 promoter (a maize polyubiquitin-like promoter), the embryo-preferred PGNpr2 promoter (maize globulin-1, Belanger and Kriz, 1991), or the endosperm-preferred PGNpr3 promoter (maize 22 kDa alpha-zein, GenBank AF090447). We tested many combinations of promoters and targeting sequences to determine the best parameters for achieving a high level of laccase expression in maize seed. The combinations included the promoters described above with targeting sites of cell wall (CW), endoplasmic reticulum (ER), vacuole and cytoplasm (Figure 1).

Table 1 Expression cassette features, event recovery and expression level of highest seed for laccase in maize

Plasmid number	Promoter	Subcellular location	Number of events	Expression level of high T_1 seed*
PGN8908	Em	CW	13	0.8
PGN8996	Em + I	CW	11	0.25
PGN7718	C	CW	15	0.19
PGN7699	C	ER	20	0.12
PGN8915	C	Cytoplasm	8	0.07
PGN8988	Em + I	CW	9	0.015
PGN9067	En	CW	17	0.0035
PGN9001	Em	ER	0	0

*Expression levels are presented as extractable laccase as a percentage of total soluble protein from individual seeds.
Abbreviations: C = constitutive promoter, Em = embryo-preferred promoter, En = endosperm-preferred promoter, CW = cell wall, ER = endoplasmic reticulum, I = intron.

Transformation of Hill (Armstrong *et al.*, 1991) maize embryos was accomplished using *Agrobacterium tumefaciens* essentially as described by Ishida *et al.* (1996). Because a selectable marker gene for herbicide resistance was present in the transformation vectors, individual transformation events were identified when they grew rapidly on bialaphos-containing medium (3 mg/L). Our target number of independent transgenic events was 10–15, and in most cases, we were able to recover this number (Table 1). In cases where fewer than 10 transgenic events were recovered, repeated attempts were made to achieve that number. However, if at least 8000 immature embryos were treated with the *Agrobacterium* strain, and transgenic event recovery remained low to none, attempts were abandoned, and the conclusion was drawn that the expression cassette was having an affect on the recovery of healthy transformation events. The most striking result was with the PGN9001 construct where no transgenic events were recovered after several thousand embryos were treated with the strain. All putative events identified from this vector turned brown and died in culture.

Expression of fungal laccase in transgenic maize seed

Approximately 10 plants per independent transformation event (10 clones) were regenerated from embryogenic calli as previously described (Hood *et al.*, 1997), and allowed to flower and set seed in the greenhouse. Because our main focus was on protein production from seed (Hood *et al.*,

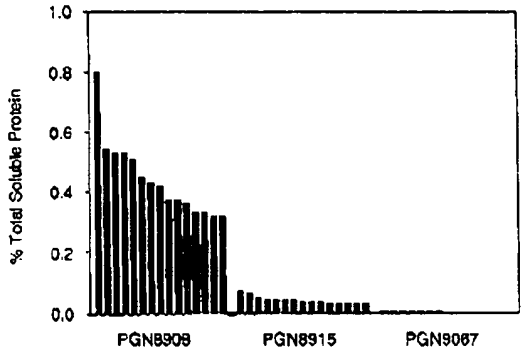

Figure 2 Expression levels of highest 15 individual T_1 seeds from each of three vectors used to generate independent transgenic events to express laccase in maize. The data for the top 15 expressing seeds from vector PGN8908 are derived from 12 plants from seven events, from vector PGN8915, 11 plants from seven events, and from vector PGN9067, eight plants from five events.

1997, 1999; Hood, 2002), laccase expression was monitored for all transgenic plants that produced seed. Several individual seeds from each plant were analysed for expression of the transgene, and several high-expressing plant lines representing multiple independent transformation events were chosen for further experiments.

Each mature laccase molecule contains four copper ions, and we have discovered that extracted laccase is not completely active without treatment with copper ions *in vitro* (M. Bailey unpublished; PCT/US01/19174). Therefore, each of the seed extracts was copper-treated before enzyme levels were measured in the activity assay. T_1 (first generation transformed) seeds were individually analysed for laccase content after extraction of total soluble proteins in 20 mM sodium acetate, pH 5.0, with 0.05% Tween-20 (SAT). Laccase in the extracts was activated by treatment with copper sulphate and analysed in a 96-well microtitre plate, using ABTS as the substrate (Bourbonnais and Paice, 1990). Laccase amounts were determined by comparison with a known amount of recombinant *Trametes* laccase as standard. Enzyme concentrations were expressed as a percentage of the soluble protein in T_1 seed (Figures 2 and 3), or as ng of laccase per mg dry weight of seed tissue in later generations.

In an effort to identify the independent transgenic events with high expression, we examined several individual seeds from the clonal plants generated from all events. We performed statistical analyses of various sample sizes to determine the number of individual seed analyses that were required to assess whether a plant from an independent transgenic event was high-expressing. A sample size of six single seeds analysed from an ear was sufficient to predict

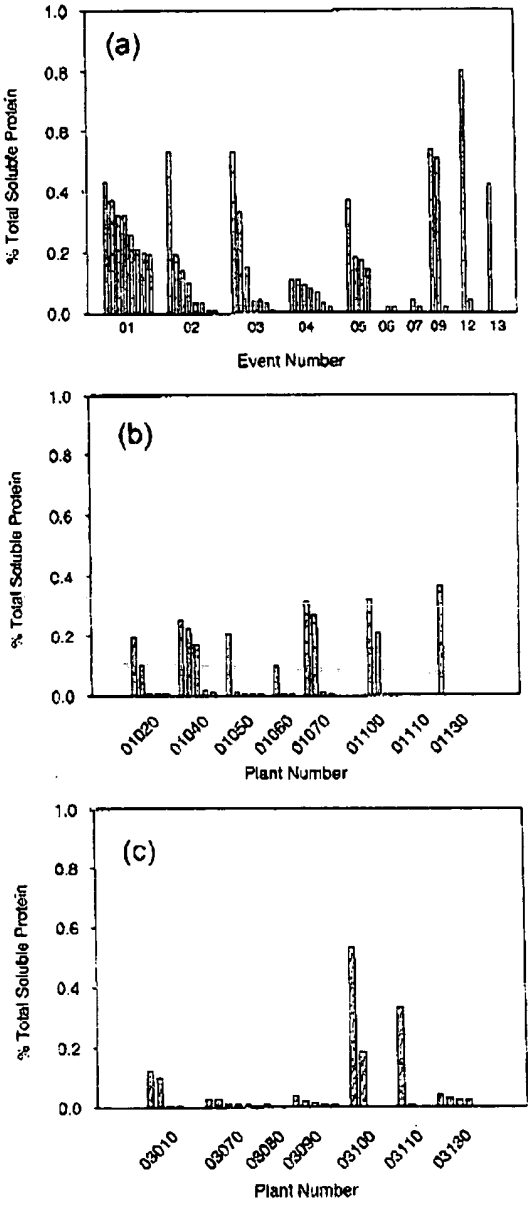

Figure 3 Between and within event variation of transgenic plants. (A) Expression of highest T_1 seed (per cent of total soluble protein) from each plant line regenerated from all events from PGN8908 – consistency of highest expressing construct. (B, C) Expression of all positive T_1 seed assayed from a single event (five seeds per plant), expressed in groups of seeds per plant line regenerated from that event. (B) Event 03. (C) Event 01.

with a 70% probability that a plant would be the highest-expressing plant within the sampled population (E. Hood, S. Woodard, C. Gates, unpublished data). Moreover, we were interested in the highest potential of individual positive T_1

seed from each plant, rather than the average expression, because the potential was what we were trying to maximize. The values for expression from individual seeds varied widely, even among positive seed from the same ear (see below). Therefore, averaging the data for T_1 seed from a single plant masked the expression potential of that plant. Thus, to identify the highest-expressing plants and therefore the highest potential for improving their expression to commercial levels, it was important to analyse and express data on an individual seed basis. Moreover, high values for individual seed were relatively consistent for a single vector. In other words, the highest single seed expression level observed for each vector (Table 1) was representative of several events generated from that vector. The data shown in Figure 2 illustrate the concept that choosing a best vector based on highest single seed was a valid method. The highest 15 single seed values from each of the three vectors shown demonstrated great similarity to one another, but showed that substantial differences existed among vectors. The difference between the highest expressing and 15th highest expressing seeds for PGN8908 was 2.3-fold, whereas the difference between the highest seed from PGN8908 and the highest seed from PGN8915 was greater than 10-fold, and from PGN9067 100-fold. This phenomenon is characteristic of more than one event from a vector. The data shown in Figure 2 represent for PGN8908: 12 plants from seven events, for PGN8915: 11 plants from seven events, and for PGN9067: eight plants from five events. Thus, some plants produced more than one seed that fell into the top-15 expressing category.

Interestingly, even though vectors can be ranked as to those that are best, intermediate or worst, substantial variability was observed among independent transgenic events from a single vector (Figure 3). Highest individual seed values for each plant derived from all independent events from vector PGN8908 are graphed from highest to lowest for each of the events recovered (Figure 3A). Almost all independent events from the PGN8908 construct produced individual plants that had a high laccase activity in the T_1 seed extract (Figure 3A). However, some independent events, namely 04, 06 and 07, produced plants that had lower levels of expression, demonstrating that variability among independent events occurred. However, in no case were transgenic events isolated that showed zero expression of the target protein, i.e. all events surviving selection also expressed the gene of interest.

Variability was also seen among plants clonally derived from each of those independent transformation events (Figure 3B and C). The high-expressing seed from each plant from event 01 varied from 0.01% TSP for plant 01110 to 0.38% TSP for plant 01130 (Figure 3B). Within event 01, only two plants yielded seeds with relatively low expression levels: 01060 and 01110. High expression levels were seen in seeds from all other 01-derived plant lines, demonstrating that this independent transgenic event had several clonal lines that were good candidates for propagation in the breeding programme. For event 03, the highest seed per line varied from 0.01% TSP for 03080 to 0.55% TSP for line 03100 (Figure 3C). Plants 03100 and 03110 had individual seed values that were much higher in expression than other plants from this event (Figure 3C). However, even among positive seed from these two high-expressing plants, a large variation was seen. For example, plant 03100 had four laccase positive seeds ranging in expression from 0.01% TSP to 0.55% TSP. Plant 03110 had three positive seeds ranging in expression from 0.01% TSP to 0.35% TSP. These results demonstrate the within-plant variability of seed that we observed for all transgenic events and plants recovered. Though transgenic events 01 and 03 were not the highest-expressing events from the PGN8908 vector, they were chosen for their overall robustness, in addition to their excellent expression levels, to continue breeding into better germplasm.

Although the amount of laccase in transgenic seed varied in the events and lines produced from each vector, we were able to determine a combination of target site and promoter, i.e. a vector, that was superior to others for driving high levels of laccase accumulation in seed tissue (Table 1). The data in Table 1 reflect the highest value detected for a T_1 seed for each of the vectors in this study. The data in Figure 2 demonstrate that the highest T_1 seed data were not anomalies and did reflect the performance of plants generated from each of the vectors.

For the constitutive promoter, the cell wall was apparently a better site for protein accumulation than the endoplasmic reticulum, which in turn was somewhat better than the cytoplasm (Table 1). This ranking existed even though the promoter should function the same in these various transformants. Plants from ER targeted events were unhealthy and died early (data not shown). The final result was that events with protein localized to the cell wall accumulated laccase to the highest expression levels. The data for seed expression from the embryo-preferred promoter also showed that the cell wall was the best site for protein accumulation (Table 1).

Introns have been shown to have a positive effect on heterologous gene expression in maize (Callis *et al.*, 1987). The version of the embryo-preferred promoter we used does not include an intron. Therefore, we chose to test it with an intron from either AdH-1 (Dennis *et al.*, 1984) or ubiquitin-1 (Christensen *et al.*, 1992). In each case the intron was fused

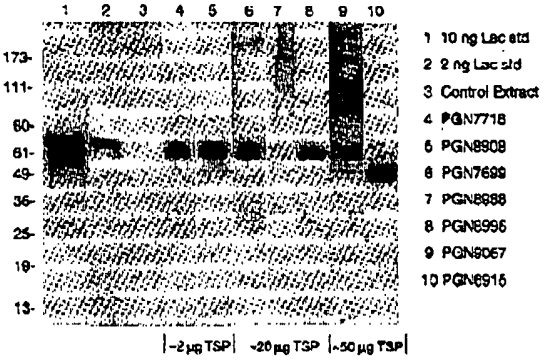

Figure 4 Western blot of representative T_1 seed extracts for each vector illustrated in Figure 1. Bands on the gels run relative to protein standards (numbers to left of blot) and all gels were run with similar conditions and thus protein sizes can be compared. Extracts in lanes 4 and 5 contain approximately 2 µg total soluble protein (TSP) as extracted from corn flour. Lanes 6, 7 and 8 have been concentrated approximately 10-fold and lanes 9 and 10 have been concentrated approximately 25-fold before loading on the gel. Laccase protein was detected using anti-laccase antibodies raised in rabbits. Secondary antibody was conjugated to horseradish peroxidase and detected with chemiluminescense.

to the 5′ leader downstream of the transcriptional start site but prior to the start of translation. Neither intron enhanced the expression of laccase from the PGNpr2 promoter. In fact, both introns actually considerably lowered the expression from the embryo-preferred promoter (Table 1). This difference could be due to differences in the 5′ untranslated region associated with each intron. We did not investigate this phenomenon further.

The most surprising result was that the endosperm-preferred promoter was not effective at driving high-level expression of laccase in endosperm (Table 1). Clearly, the cell wall target was an effective one for protein accumulation (see above), but was not a good combination with this endosperm-preferred promoter.

To assess the quality of the protein extracted from transgenic seed, extracts from independent events generated from the vectors described in Figure 1 were analysed by Western blot (Figure 4). The blots were developed with antilaccase antibodies raised in rabbits whose sera had low levels of cross-reactivity with native corn seed proteins. The T_1 seed extracts from non-cytoplasmic events contained two bands that were similar in molecular weight (≈ 63 and 65 kDa) to the two major bands visualized in the *Trametes* control (64 and 66 kDa; Figure 4). These data also showed that the secreted and ER forms of the protein were larger than the cytoplasmic version of the protein. To determine if the non-cytoplasmic forms were glycosylated (data not shown), the oligosaccharides on PGN8908-derived laccase were converted to aldehydes by periodate treatment and detected with an alpha fuchsin stain (GelCode Glycoprotein Stain, Pierce Biotechnology). They were positive, most likely accounting for the apparent differences in molecular weight (compare lanes 4–9 with lane 10). Indeed the size of the cytoplasmic version (PGN8915) was predicted by the amino acid sequence from the gene (55 kDa). The amount of protein recovered in the activity assay correlated well in every case with the amount seen on a Western blot (Figure 4).

From the data shown in Table 1 and Figures 2–4, we concluded that PGN8908 was the best vector to yield the highest individual seed expression level. Therefore, we continued with an in-depth analysis of events and plants from this construct.

Cellular and molecular characterization of selected transgenic lines

Seeds from the highest expressing constructs from the constitutive and embryo-preferred promoters, PGN7718 and PGN8908, have a smoky brown appearance, although this is most pronounced in PGN8908-derived events (Figure 5A and B). This morphology was observed to segregate in a 1 : 1 fashion in T_1 seed and later generations that were produced by out-crossing. Extracts of smoky and yellow seeds were made separately to demonstrate that the dark colour correlated with laccase activity (data not shown). The colour appeared to be darkest at the dent of the endosperm and only slightly coloured the embryo, even though events were derived from constructs with embryo-preferred promoters.

We were interested in the localization of laccase activity in the seed of events derived from several constructs. Therefore, cross-sections of seeds from these constructs were stained for laccase activity using diaminofluorene (DAF). Diaminofluorene, a colourless substance in solution, is converted to a dark blue insoluble product through the activity of laccase. This is a qualitative method that shows the relative laccase activity in several transgenic lines, judged subjectively by the intensity of and time for colour development. Moreover, it demonstrates tissue-localized activity, although it is not sensitive enough to indicate a subcellular locale (Figure 5). Representative seeds from PGN7699, PGN7718, PGN8908 and PGN9057 were treated with DAF and only those from PGN7718 and PGN8908 showed a significant blue colour (Figure 5C and E). Staining intensity between these two constructs roughly correlated with extractable laccase amounts – 0.2% and 0.8% total soluble protein, respectively (Table 1). For the PGN8908 lines, DAF resulted in intense staining and thus showed a high laccase activity almost exclusively in the embryo (germ), although it was also present in the aleurone

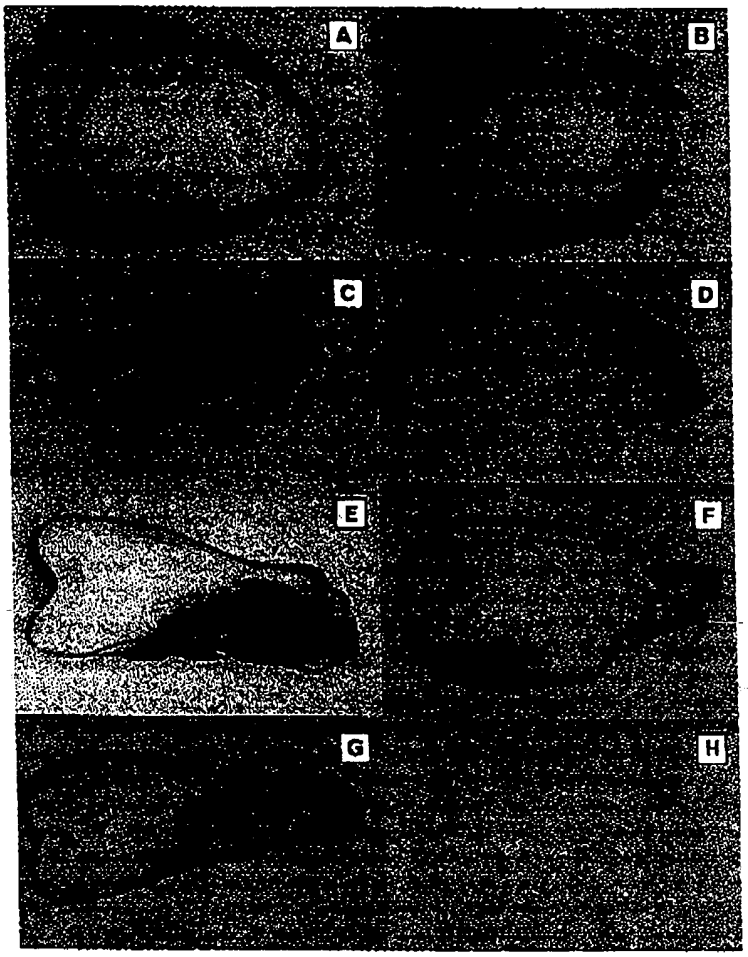

Figure 5 Laccase confers physical characteristics on seed derived from the transgenic plants expressing the laccase I gene of *T. versicolor*. (A, B) Colour morphology of (A) yellow (laccase negative) and (B) smoky (laccase positive) seed. (C–F) DAF staining of cross-sections of seeds from PGN7718, PGN7699, PGN8908 and control seeds, respectively. DAF positive staining is dark blue and primarily in the embryo. (G, H) Phloroglucinol staining of sections of PGN8908 and control seeds. Note pink to brown colour in the embryo of PGN8908 seed.

layer. The aleurone layer activity appears to be a native laccase or other enzyme activity, since control tissue also has this staining pattern (Figure 5F). The PGN7718 line primarily showed activity in the embryo as well, confirming our previous observation that the constitutive promoter directs expression preferentially to the embryo of the seed (Hood *et al.*, 1997, 1999). Lines from PGN7699 (Figure 5D) and PGN9067 (data not shown) showed little to no colour development, correlating with lower amounts of extractable laccase activity (Table 1).

The germination of smoky seed was poor, less than 50% in most cases (Table 2). We hypothesized that because laccase has been implicated in lignin formation (Whetten and Sederoff, 1995), perhaps the embryos were becoming lignified in the lines expressing high laccase activity, which was possibly inhibiting radical and shoot elongation. Indeed, phloroglucinol staining of control or yellow seed showed lignin only in vascular tissue near the attachment point of the seed to the cob (Figure 5H). In contrast, smoky seed stained strongly for lignin (pink to brown colour) in the entire embryonic axis and scutellum (Figure 5G). We have observed slow emergence and stunting and curling of the radical when these seeds are germinated *in vitro*.

Table 2 Germination of laccase-positive seed in various genetic backgrounds

Germplasm type	Germination
Elite 1, lancaster	40%
Elite 2, stiff stalk	39%
High oil corn	61–75%
Opaque 2	2%

The high expression levels achieved in events 01 and 03 from PGN8908 were not due to multiple integration events of the gene. *Nco*I digests (Figure 6) indicated that the internal laccase fragment inserted into the plants was the same size

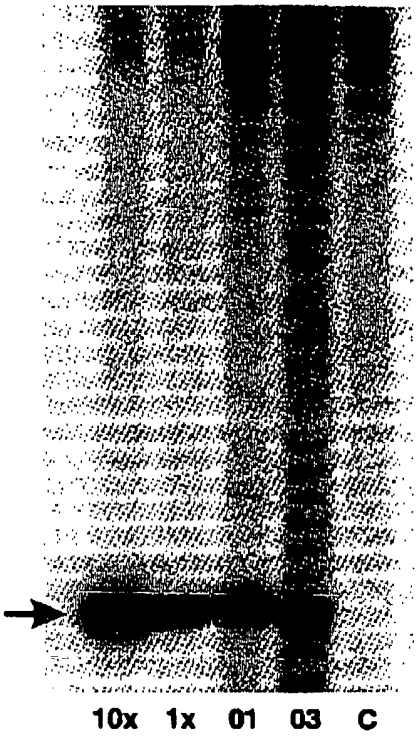

Figure 6 Southern blot of PGN8908 events targeted for commercialization. Control lane (1x and 10x) is PGN8908 DNA digested with *Hpa*I and loaded at a level equivalent to a single copy and 10 copies of the laccase gene in maize DNA. Lanes 01 and 03 contain DNA from events 1 and 3 of the PGN8908 plasmid, respectively. C is DNA from a non-transformed control maize plant. Plant DNA was digested with *Nco*I. Blot was probed with the laccase I gene labelled with ^{32}P.

as in the parent plasmid (1.9 kb). Plant DNA from events 01 and 03, when cut with *Sac*I, showed that event 01 has two fragments of 6.4 and 5.0 kb, the predicted sizes for a tandem repeat of the T-DNA insertion in a single insertion site. Event 03 had a single *Sac*I fragment at greater than 23 kb (data not shown). The laccase levels in event 01 plants were not double those of 03, indicating that expression was not simply related to copy number.

Genetic manipulation of the transgenic plants

The Hill maize line that was used in tissue culture for plant transformation shows poor agronomic characteristics and is not high-yielding in the field. However, one of the most important goals for industrial protein production is a yield near that of commercial corn lines. Thus, the agronomic quality of early transgenic material must be improved through an intense breeding programme. T_1 seed from selected high-laccase-expressing independent lines was planted in nurseries

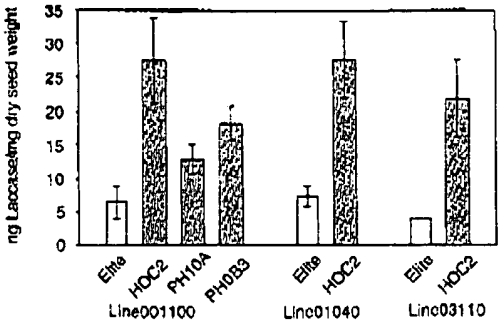

Figure 7 Effect of germplasm on expression of laccase. Three transgenic lines from two events: 0110, 0104 and 0311, were crossed to elite germplasm, or to several high oil lines, HOC2, PH10A or PH0B3. Note that the high oil germplasm allows higher expression of laccase than elite germplasm.

and crossed to elite inbreds. The goal was to develop high-yielding hybrids with good agronomic qualities. Early generations yielded lines with increased expression, but agronomic qualities, including germination, were not improving. Therefore, crosses to several germplasm pools were done in an attempt to recover healthier material for propagation. Of the germplasm tested, only the high oil lines generated material that allowed the recovery of high expression (Figure 7), high germination rates (Table 2), and acceptable yields. Several lines from events 01 and 03 showed these traits – line 01100, line 01040 and line 03110, for example. In each of these cases, the transgenic line was crossed to an elite inbred, or to a high oil germplasm. The transgenic laccase expression in these backgrounds was significantly higher than laccase expression in the elite backgrounds (Figure 7). In addition, the germination of seed that had been crossed once to a high-oil germplasm showed an improved germination to over 70% (Table 2), even though the extractable laccase expression in the high oil germplasm was higher than in elite germplasm.

For laccase line 01100, a single plant derived from event 01, we improved the expression of extractable laccase by nearly 20-fold from generation T_2 to T_6 (4 ng laccase/mg tissue dry weight to 70 ng laccase/mg tissue dry weight, Figure 8). The progression was that in each generation, the highest expressing ears showing agronomic promise were selected and the seed replanted from those ears in subsequent nurseries. After pollination, maturation and harvest, 50 seed from each progeny ear were combined, ground and analysed for expression levels of extractable laccase. Only those showing improvement in the amount of extractable laccase were selected for replanting. At each generation, approximately the top 10% of lines were replanted for the breeding programme. In the case of laccase, some of the best expressing

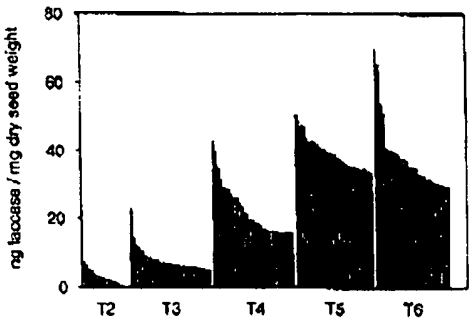

Figure 8 Improvement of laccase expression in the LCG0110 line over five generations. The lines from each generation with the highest levels of extractable laccase were selected for replanting in the subsequent generation. The top 10% of lines were propagated and the progeny analysed.

lines produced seed that would not germinate in the field. Thus, the selection of the highest expressing healthy lines took two to three extra generations, during which time they were being bred into high oil germplasm for the recovery of healthy plants. These data show the power of genetics in improving the agronomic quality and expression of the target protein for commercial purposes.

Discussion

Industrial proteins produced in plants offer a unique opportunity for the industrial scale production of enzymes and functional proteins at competitive prices and yields that will address markets that cannot be addressed today. As a demonstration of the power of this technology, we have expressed the oxidative enzyme laccase at high expression levels and thus opened the possibility of addressing such large markets as pulp bleaching, textile bleaching and enzymatic remediation of waste streams.

To produce industrial enzymes at a cost-competitive price, we estimate that the expression levels in whole grain must be near 0.1% of dry weight. This translates to ≈ 0.5% dry weight in a germ fraction, an amount potentially viable for industrial applications of laccase (Bailey *et al.*, submitted). In addition, laccase generally requires a mediator for its activity, and components present naturally in corn seed may provide such a mediating activity. Although the extractable levels for T_6 generation seed are = 70 ng/mg in the highest ear bulks, the activity in defatted germ flour is much higher, i.e. = 0.5% dry weight (Bailey *et al.*, submitted), estimated from an activity assay.

The recovery of high laccase protein accumulation depends on the promoter and targeting site. We tested three quite distinct promoters and three subcellular locations to determine which sites would make the best locations for a high-level accumulation of this protein. As shown in Table 1, the embryo-preferred expression in which the protein is targeted to the cell wall provides the best location for this protein, in that these parameters allow the recovery of the highest amounts of extractable laccase. PGN8908 yielded lines that express fourfold higher extractable laccase than the constitutive promoter with the cell wall target, a significant difference for the recovery of commercial laccase preparations.

The constitutive expression of laccase, such as is present in PGN7718-derived events, even though cell wall targeted, caused numerous plant health problems that prevented effective seed increases of the material in the field. Because of leaf burning and stunting of the plants, the high-expressing PGN7718-derived plants died in the field, and could not be propagated. Thus, the constitutive expression of laccase is not practical. Surprisingly, the endosperm expression was not practical either, in that only quite low-expressing events were recovered. We believe this was a result of the promoter rather than the targeting site, because we have tried this promoter in combination with several other genes and targeting sites with equally poor expression (data not shown).

Subcellular locations other than the cell wall for accumulation of this protein were not successful. Possible reasons for this might include protein instability or high protein accumulation in the cytoplasm and in the ER that proved detrimental. Indeed, several attempts were made to recover plants from ER targeted events without success. The few plants recovered from events generated from constructs using constitutive promoters and ER targeting sequences died when they were transferred from the low light of culture into the greater light of the growth rooms prior to transfer to the greenhouse. No events were ever recovered from ER targeted protein expressed with the embryo-preferred promoter (PGN9001, Table 1), possibly because the protein was expressed and its effects were lethal at the tissue culture stage. All recovered cytoplasmic events exhibited a low expression in the seed.

Events from all vectors showed variation among vectors, among events from each vector and among plants from each event. However, the PGN8908 vector utilizing the embryo-preferred promoter and cell wall target was clearly the best plant transcription unit, as several high-expressing events were generated from this construct. A large variation was also seen among individual plants from a single transgenic event, illustrating the concept of a unique plant from a transgenically clonal population. This is most likely due to unique genetic combinations in the T_1 seed resulting from out-crossing with inbred pollen and segregation of the genes from the hybrid raw material used for transformation.

The concept of improving transgene expression over generations using genetics and selection is foreign to most researchers. However, this is the result we have obtained with numerous proteins in our breeding programme. The 'best-expressing' individuals can be perpetuated in subsequent generations in the field (refer to Figure 8) with coincident improvements in transgene expression. Improvements achieved between generations T_5 and T_6 were less dramatic than those seen in previous generations. This was most likely a result of these plants having reached maximum achievable expression for this enzyme, and only a few lines were able to demonstrate the increased levels in T_6. Those highest lines were chosen for further propagation. In addition from genetic improvement through breeding to commercial inbreds, unique germplasm, such as high oil, can be used to increase yield, influence germination and maintain or improve transgene expression. With regard to germination, we hypothesize that the high oil lines may provide substrate (i.e. oil) for the laccase-generated free radicals to act upon, thereby preventing the accumulation of free radicals that alter seed physiology, such as increased liginification. The seed can then germinate and the plants can survive in the field.

Protein concentration in the target material can make or break commercial potential. This minimum useful concentration is determined by the sale price of the protein, and thus commercial levels of expression are different for different product categories. Expression levels of laccase that are commercially attractive are produced by a combination of the above factors. While levels at about 0.01% of dry weight are commercially useful, expression levels of 0.1% dry weight would be even more attractive, as they would allow recovery of 1 g active ingredient from 1 kg of corn. This target level requires significant technological development for enzymes. The experiments described here have begun to address these issues.

Acknowledgements

We gratefully acknowledge the artistic help of Ms Christine Stetter, College Station, TX, for completion of halftone figures.

References

An, G., Mitra, A., Choi, H.K., Costa, M.A., An, K., Thornburg, R.W. and Ryan, C.A. (1989) Functional analysis of the 3' control region of the potato wound-inducible proteinase inhibitor II gene. *Plant Cell*, **1**, 115–122.

Armstrong, C.L., Green, C.E. and Phillips, R.L. (1991) Development and availability of germplasm with high Type II culture formation response. *Maize Genet Coop Newsl.* **65**, 92–93.

Bailey, M., *et al.* (in press) Active recombinant laccase from maize seed and its utility as a binding agent. *Appl. Microbiol. Biotechnol.*, submitted.

Belanger, F.C. and Kriz, A.L. (1991) Molecular basis for allelic polymorphism of the maize Globulin-1 gene. *Genetics*, **129**, 863–872.

Bell, D.J., Hoare, M. and Dunnill, P. (1983) The formation of protein precipitates and their centrifugal recovery. *Adv. Biochem. Engng.* **26**, 2–68.

Bourbonnais, R. and Paice, M.G. (1990) Oxidation of non-phenolic substrates. An expanded role for laccase in lignin biodegradation. *FEBS Lett.* **267**, 99–102.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254.

Callis, J., Fromm, M. and Walbot, V. (1987) Introns increase gene expression in cultured maize cells. *Genes Dev.* **1**, 1183–1200.

Christensen, A.M., Sharrock, R.A. and Quail, P.H. (1992) Maize polyubiquitin genes: structure, thermal perturbation of expression and transcript splicing, and promoter activity following transfer to protoplasts by electroporation. *Plant Mol. Biol.* **18**, 675–689.

Dennis, E.S., Gerlach, W.L., Pryor, A.J., Bennetzen, J.L., Inglis, A., Llewellyn, D., Sachs, M.M., Ferl, R.J. and Peacock, W.J. (1984) Molecular analysis of the alcohol dehydrogenase (Adh1) gene of maize. *Nucl. Acids Res.* **12**, 3983–4000.

Dudley, J.W. and Lambert, R.J. (1992) Ninety generations of selection for oil and protein in maize. *Maydica*, **37**, 81–87.

Hiei, Y., Ohta, S., Komari, T. and Kumashiro, T. (1994) Efficient transformation of rice (*Oryza sativa* L.) mediated by Agrobacterium and sequence analysis of the boundaries of the T-DNA. *Plant J.* **6**, 271–282.

Hood, E.E. (2002) From green plants to industrial enzymes. *Enzyme Microbial Technol.* **30**, 279–283.

Hood, E.E., Helmer, G.L., Fraley, R.T. and Chilton, M.D. (1986) The hypervirulence of *Agrobacterium tumefaciens* A281 is encoded in a region of pTiBo542 outside of T-DNA. *J. Bacteriol.* **168**, 1291–1301.

Hood, E.E. and Howard, J.A. (1999) Plant-based production of xenogenic proteins. *Agro-Food-Industry Hi-Tech.* **10**, 382–386.

Hood, E.E. and Jilka, J.M. (1999) Plant-based production of xenogenic proteins. *Curr. Opin. Biotechnol.* **10**, 382–386.

Hood, E.E., Kusnadi, A., Nikolov, Z. and Howard, J.A. (1999) Molecular farming of industrial proteins from transgenic maize. *Adv. Exp. Med.* **464**, 127–147.

Hood, E.E., Witcher, D.R., Maddock, S., Meyer, T., Baszczynski, C., Bailey, M.R., Flynn, P., Register, J., Marshall, L., Bond, D., Kulisek, E., Kusnadi, A., Evangelista, R., Nikolov, Z., Wooge, C., Mehigh, R.J., Herman, R., Kappel, W.K., Ritland, D., Li, C.-P. and Howard, J.A. (1997) Commercial production of avidin from transgenic maize: Characterization of transformant, production, processing, extraction and purification. *Mol. Breed.* **3**, 291–306.

Ishida, Y., Saito, H., Ohta, S., Hiei, Y., Komari, T. and Kumashiro, T. (1996) High efficiency transformation of maize (*Zea mays* L.) mediated by *Agrobacterium tumefaciens*. *Nat. Biotechnol.* **14**, 745–750.

Lehninger, A.L. (1970) *Biochemistry*, 2nd edn. New York: Worth Publishers Inc.

Ong, E., Pollock, W.B. and Smith, M. (1997) Cloning and sequence analysis of two laccase complementary DNAs from the ligninolytic basidiomycete Trametes versicolor. *Gene*, **196**, 113–119.

Rheinhammar, B. (1984) Laccase. In: *Copper Proteins and Copper Enzymes* (Lonti, R., ed.), pp. 2–35. Boca Raton, FL: CRC Press Inc.

Rogers, J.C. (1985) Two barley alpha-amylase gene families are regulated differently in aleurone cells. *J. Biol. Chem.* **260**, 3731–3738.

Southern, E.M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98**, 503–517.

Whetten, R. and Sederoff, R. (1995) Lignin biosynthesis. *Plant Cell*, **7**, 1001–1013.

White, J., Chang, S.-Y., Bibb, M.J. and Bibb, J.M. (1992) A cassette containing the bar gene of *Streptomyces hygroscopicus*: a selectable marker for plant transformation. *Nucl. Acids Res.* **18**, 1062.